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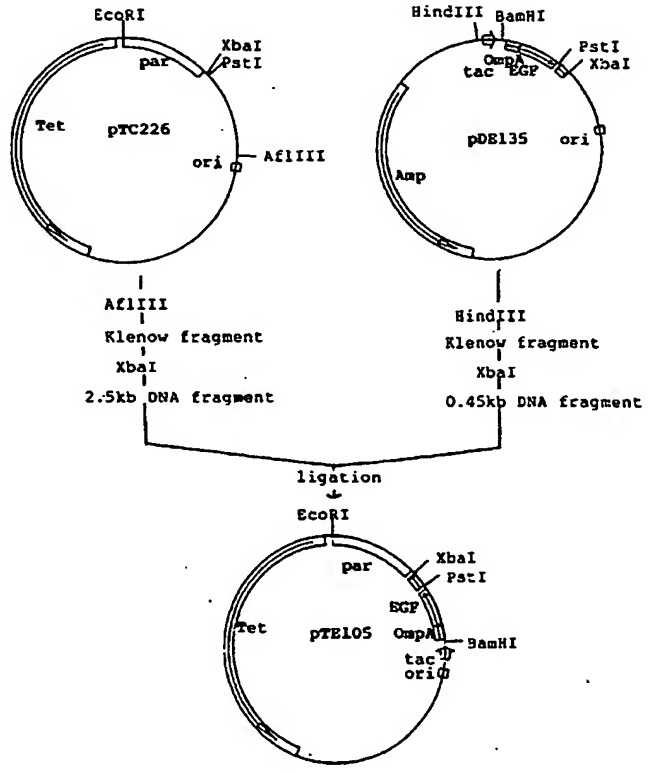
(54) Title: A NOVEL GENE CODING HUMAN EPIDERMAL GROWTH FACTOR AND PROCESS FOR PREPARING THE SAME

(57) Abstract

The present invention relates to a novel gene coding human epidermal growth factor ("hEGF") and a process for preparing the same employing a recombinant expression vector therefor. The hEGF gene of the invention is designed to contain codons ubiquitous in E. coli and the following restriction sites: HpaI at the 5' terminal, PstI at the 3' terminal and Bpu1102I, NsiI, MluI, Eco47III and AflII at a regular manner within its internal sequence. The present invention also provides a process for preparing REGF by employing an expression vector pTE105 for hEGF, which contains expression cassette comprising Omp A leader sequence, translation termination sequence and transcription termination sequence and hEGF gene; and, replication origin of pUC19, tetracycline-resistant marker and a par site for stabilization in E. coli. The hEGF is produced massively in E. coli transformed with the pTE105 (KCCM 10027).



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A NOVEL GENE CODING HUMAN EPIDERMAL GROWTH FACTOR AND PROCESS FOR PREPARING THE SAME

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a novel gene coding human epidermal growth factor and a process for preparing the same, more specifically, to a novel human epidermal growth factor gene and a process for preparing the same employing a recombinant expression vector therefor.

Description of the Prior Art

It has been known that human epidermal growth factor (hereinafter referred to as 'hEGF') is a polypeptide hormone consisted of 53 amino acids and 3 disulfide bridges[see: Cohen, S., J. Biol. Chem., 237:1555-1562(1962); Savage, C.R., Jr. et al., J. Biol. Chem., 248:7669-7672(1973); Savage, C.R., Jr. et al., J. Biol. Chem., 247:7612-7621 (1972)], and plays an important role on the growth control in mammalian cells, inter alia epidermal and epithelial cells on molecular level[see: Sporn, M.B. et al., Nature (London), 313:745-747(1985); Sporn, M.B. et al., N. Engl. J. Med., 303:878-880(1980)] and the treatment of injury as well [see: Buckley, A. et al., Proc. Natl. Acad. Sci., USA, 82: 7340-7344(1985)]. Further, it has been reported that the hEGF can be applied in the treatment of a stomach ulcer, grounded on the facts that it represses secretion of gastric acid into stomach[see: Gregory, H., J. Cell Sci. Suppl., 3: 11-17(1985)].

Under the circumstances, studies on the mass production of the hEGF has been actively carried out, since Starkey et al. had reported the biochemical property of hEGF purified from human urine[see: Starkey, R. H. et al.,

Science, 189:800(1975); Cohen, S. et al., Proc. Natl. Acad. Sci., USA, 72:1317(1975)]; and, several researchers have accomplished cloning of hEGF gene by the recombinant DNA technology in a successful manner[see: Smith, J. et al., Nucleic Acids Res., 10:4467-4482(1982); Urdea, M. S . et al., Proc. Natl. Acad. Sci., USA, 80:7461-7465(1983); Oka, T. et al., Proc. Natl. Acad. Sci., USA, 82:7212-7216(1985)]. The prior art, however, have not provided the hEGF to the level of industrial application owing to its low activity and productivity.

Accordingly, studies on the elevation of activity and productivity in hEGF manufacture have been carried out, which are primarily concentrated on the preparation of the nucleotide sequence of hEGF gene efficient for its massive production and the expression vector whose regulatory function is strengthened.

SUMMARY OF THE INVENTION

In accordance with the present invention, the inventors synthesized a novel hEGF gene and a novel expression vector therefor which expresses hEGF in a massive manner and developed a process for preparing hEGF therefrom.

A primary object of the invention is, therefore, to provide a novel hEGF gene which is designed and chemically synthesized for the purpose of massive expression of hEGF in E. coli.

Another object of the invention is to provide a novel process for preparing hEGF from a recombinant expression vector comprising said hEGF gene and regulatory sequence.

BRIEF DESCRIPTION OF THE DRAWINGS

The above and the other objects and features of the present invention will become apparent from the following descriptions given in conjunction with the accompanying drawings, in which:

Figure 1 depicts sequence of designed hEGF gene of the invention;

Figure 2A depicts 10 oligonucleotides synthesized;

Figure 2B depicts assembly pattern of synthesized 10 oligonucleotides of Figure 2A;

Figure 3 depicts construction strategy of pUE118;

Figure 4 is a photograph showing agarose gel electrophoresis pattern of pUE118 carrying hEGF gene digested with restriction enzymes;

Figure 5 depicts the Omp A leader-universal translation termination-Trp A transcription termination sequence;

Figure 6 depicts construction strategy of pDT420;

Figure 7 depicts construction strategy of pDE135;

Figure 8 depicts construction strategy of pTC108;

Figure 9 depicts construction strategy of pTC226;

Figure 10 depicts construction strategy of pTE105;

Figure 11A is a photograph showing SDS-PAGE pattern of hEGF expressed in E. coli JM101 harboring pTE105;

Figure 11B is a photograph showing the Western blot analysis of hEGF expressed in E. coli JM101 harboring pTE105;

Figure 12 is a photograph showing SDS-PAGE pattern of hEGF expressed from E. coli JM101 harboring pTE105 in each purification step;

Figure 13 is a photograph showing SDS-PAGE pattern of purified hEGF from E. coli JM101 harboring pTE105;

Figure 14 is a chromatogram of purified hEGF by reverse phase HPLC; and,

Figure 15 is a photograph showing isoelectric focusing analysis of purified hEGF from E. coli JM101 harboring pTE105.

DETAILED DESCRIPTION OF THE INVENTION

The inventors synthesized a hEGF gene designed to maximize expression of the gene which satisfies the following requirements: first, hEGF expressed therefrom shall be the same as natural huamn EGF in light of amino acid sequence and protein structure; secondly, nucleotide sequence shall be designed in consideration of the codons ubiquitous in E. coli; thirdly, the portion of secondary structure of mRNA transcribed therefrom shall be minimized; fourthly, many unique restriction sites shall be positioned as far as possible.

In this connection, the hEGF gene of the invention is designed, based on the previously known hEGF sequence in the art[see: Gregory, H., Nature, 257:325(1975)]. First of all, the inventors synthesized 10 complementary oligonucleotides by using automatic Nucleotide Synthesizer in accordance with solid-phase phosphate triester method[see: Narang, S. A., Synthesis and Applications of DNA and RNA, Academic Press, 1987]; and, to obtain the full hEGF gene, shot-gun ligation method is employed.

The hEGF of the present invention contains the following restriction sites: HpaI at the 5′ terminal, PstI at the 3′ terminal and Bpu1102I, NsiI, MluI, Eco47III and AflII within its internal sequence. Introduction of the hEGF gene to pUC18 which is a starting vector of the invention, produces the above 7 specific sites which exist as unique restriction sites in the recombinant vector. The fact that said unique sites are cleaved by their specific restriction enzymes and positioned at a regular interval, allows practical availability of the gene for mutagenesis induction, which assures high activity and stability of hEGF. Furtheron, the existence of HpaI restriction site at 5′ terminal allows production of intact hEGF free of fusion protein from the expression vector therefor.

The hEGF gene of the invention is inserted into pUC18 digested with SmaI and PstI, and plasmid thus obtained is

named pUE118. E. coli JM109 is transformed with pUE118 according to Hanahan's method[see: DNA Cloning Vol.I: A Practical Approach, IRL Press, 1985, pp 109-135], and this transformant is deposited with Korean Culture Collection of Microorganisms(KCCM) located in Department of Food Engineering, College of Eng., Yonsei University, Sodaemun-gu, Seoul 120-749, Korea on April 9, 1993 in the name of E. coli DW/BT-2040(KCCM 10026).

On the other hand, it has been well known that regulation of protein expression corresponding to growth pattern of microorganism is very important in massive production of protein. In this connection, tac promoter [see: de Boer et al., DNA, 2:231-235(1983); Amann et al., Gene, 25:167-178(1983)] is introduced to the expression vector of the invention. Since the tac promoter has continuous two ribosome binding sites at the downstream[see: Shine and Dalgarno, Proc. Natl. Acad. Sci., USA, 71:1342 (1974)], it initiates the translation of the hEGF gene efficiently.

For the accurate and efficient expression and secretion of hEGF, the expression vector of the invention employs the following sequences: (1) Omp A leader sequence[see: von Gabain, A. et al., Proc. Natl. Acad. Sci., USA, 80:653-657 (1983)]; (2) universal translation termination sequence[see: P. Singleton and D. Sainsbury, Dictionary of Microbiology and Molecular Biology, 2nd Ed., Wiley, 383, 1987]; and, (3) trp A transcription termination sequence[see: Christie, G. E. et al., Proc. Natl. Acad. Sci., USA, 78:418(1981)]. Further, the expression vector contains par site for the stability in E. coli[see: Austin and Abeles, J. Mol. Biol., 169:373-387(1983)].

"Omp A leader-universal translation termination-trp A transcription termination sequence" is designed and synthesized to insert hEGF gene of the invention, which comprises restriction sites of BamHI at 5' terminal and XbaI at 3' terminal, and NaeI and PstI between Omp A leader and universal translation termination sequence.

On the other hand, it has been known that secretion of the expressed protein is decreased when ampicillin-resistant marker which codes β-lactamase, a secretory protein, is used to screen E. coli containing the vector comprising said marker, grounded on the fact that the two proteins, i.e., interested protein and β-lactamase, compete in the course of secretion[see: A. Oka, et al., J. Mol. Biol., 147:217 (1981)]. Under the circumstance, tetracycline-resistant marker coding intracellular protein, which brings about high secretion of expressed protein by avoiding said competition, is employed in the invention instead of the ampicillin-resistant marker. As a result, it is clearly guaranteed that the expression vector of the invention expresses and secretes hEGF in high yield, and is stable in E. coli.

A commercially available plasmid pDR540 is digested with PvuII and then ligated with XbaI linker; and, double digestion with XbaI and BamHI is carried out. 2.4kb of DNA fragment thus obtained is ligated with "Omp A leader-universal translation termination-Trp A transcription termination sequence"; and named pDT420.

Double digestion of pDT420 with NaeI and PstI produces a cleavage between Omp A leader and universal translation termination sequence; and, pUE118 carrying hEGF gene of the invention is digested with HpaI and PstI to obtain hEGF gene. Then, the obtained hEGF gene is ligated with the pDT420 digested with NaeI and PstI, and the resultant is named pDE135.

Plasmid pUC19 known in the art is digested with DraI and EcoRI to obtain 1.2kb DNA fragment whose two ends are blunt-ended and cohesive for EcoRI, respectively; and, pBR322 is digested with AvaI and blunt-ended with Klenow's fragment, and digestion with EcoRI is followed to obtain 1.4kb DNA fragment. The above 1.4kb and 1.2kb DNA fragments are ligated with T_4 DNA ligase, and is named pTC108. As a result, pTC108 comprises tetracycline-resistant marker, multiple cloning site and replication origin of pUC19.

A par site is introduced for the stability of

expression vector in transformant and exact separation of plasmids after cell division. Plasmid pTC108 is digested with EcoRI and SmaI. On the other hand, pSC101 is digested with AvaI to obtain 3.3kb DNA fragment, which is blunt-ended with Klenow's fragment and then ligated with EcoRI linker and followed by digestion with EcoRI. The 3.3kb DNA fragment containing sticky end of EcoRI site is digested with HincII to obtain 0.37kb DNA fragment containing par site. This 0.37kb DNA fragment is ligated to the pTC108 digested with EcoRI and SmaI by T_4 DNA ligase, and the ligated plasmid is named pTC226.

Plasmid pTC226 is digested with AflIII, blunt-ended with Klenow fragment and digested with XbaI in a serial manner to obtain 2.5kb DNA fragment. On the other hand, pDE135 is digested with HindIII, blunt-ended with Klenow fragment and digested with XbaI in a serial manner to obtain 0.45kb DNA fragment. The 2.5kb and 0.45kb DNA fragments are ligated with T_4 DNA ligase, and the ligated plasmid is named pTE105.

E. coli JM101 is transformed with pTE105, and the transformant is deposited with Korean Culture Collection of Microorganisms(KCCM) located in Department of Food Engineering, College of Eng., Yonsei University, Sodaemun-gu, Seoul 120-749, Korea on April 9, 1993 in the name of E. coli DW/BT-2042(KCCM 10027).

Transformants E. coli DW/BT-2042 are grown in LB media and expression of hEGF therefrom is determined by 15% SDS-PAGE and Western blot analysis by employing commercially available hEGF(Amersham, ARN 5100, UK) as standard. The amount of expressed hEGF is determined by hEGF receptor binding analysis employing A431 cell line(ATCC CRL 1555). Culturing said transformant for 30hrs gave 343.5mg/L of hEGF, where most of the expressed hEGF was secreted out of cytosol.

The present inventors isolated hEGF from the culture, while reducing contamination of other celluar proteins and endotoxins, by employing a series of chromatographic

purification methods, i.e., Amberchrom CG71 chromatography, Q-Sepharose anion exchange chromagraphy and reverse phase C_{18} preparative HPLC. The purity of purified hEGF is determined by analytical HPLC in accordance with modified method of Hayashi et al's[see: Hayashi, T. et al., Anal. Sci., 3: 445-449(1987)].

The present invention is further illustrated in the following examples, which should not be taken to limit the scope of the invention.

Example 1: Design of hEGF gene

The hEGF gene was firstly designed, based on the hEGF amino acid sequence and Grantham et al's study on the frequency of codon usage in E. coli[see: Grantham et al., Nucleic Acid Res., 9:243-274(1981)]. Then, sequence which does not cause formation of secondary structure of mRNA transcribed therefrom was selected, while examining whether the gene contains codons ubiquitous in E. coli and causes formation of secondary structure of mRNA or not, by employing PC-FOLD(Version 2.0) program[see: Turner, D. et al, Cold Spring Harbor Symp. Quant. Biol., 52:123(1987)]. The hEGF sequence thus designed carries the following two restriction sites to guarantee accurate insertion, isolation and manipulation of hEGF gene: HpaI at the 5' terminal; PstI at the down stream of translational termination codon. The hEGF sequence futher comprises many unique restriction sites at a regular basis as followings:

HpaI-22bp-Bpu1102I-39bp-NsiI-25bp-MluI-
35bp-Eco47III-21bp-AflII-32bp-PstI

The hEGF sequence designed in the invention is disclosed in Fig. 1, which shows positions of restriction sites: HpaI at the 5' terminal, PstI at the 3' terminal, and Bpu1102I, NsiI, MluI, Eco47III and AflII.

Example 2: Synthesis of oligonucleotides

The hEGF gene designed in Example 1 was chemically synthesized. First of all, 10 oligonucleotides consisted of 29mer to 41mer were synthesized in a separate manner. They are disclosed in Fig. 2A: in this connection, C1(30mer), C2 (35mer), C3(29mer), C4(39mer) and C5(41mer) oligonucleotides have the same sequence as that of mRNA transcribed from the corresponding hEGF sequence; and, N1(29mer), N2(38mer), N3(29mer), N4(36mer) and N5 (38mer) oligonucleotides were complementary to the C5, C4, C3, C2 and C1 oligonucleotides, respectively[see: Fig. 2B]. Each oligonucleotide was synthesized by automatic Nucleotide Synthesizer(Pharmacia LKB Biotechnology, Uppsala, Sweden).

Example 3: Isolation and sequencing of oligonucleotides

Oligonucleotides thus synthesized were separated on the silica matrix, through treatment with TTD solution (thiophenol/triethylamine/dioxane = 1/2/2, v/v) and washing with methanol, ethanol and strong ammonia water in a serial manner. The solution containing oligonucleotide thus separated was subjected in strong ammonia water at 50°C for 12hrs to remove protecting group; and concentration under vacuum condition was followed to the volume of 0.5ml, together with removal of gas. The oligonucleotide solution thus concentrated was applied on SEP-PAK cartridge(Waters Inc., MA, USA), and elution was made with acetonitrile/ triethylamine solution to obtain partially purified oligonucleotide. Then, electrophoresis on 15% denatured polyacrylamide gel(TE-boric acid (pH 8.3) with 8M urea) was carried out and location of oligonucleotide in the gel was determined by the irradiation of ultraviolet rays. The gel corresponding to oligonucleotide band was cut out, oligo-nucleotide was eletroeluted and salts were removed on SEP-PAK cartridge connected with injector by eluting with acetonitrile/triethlamine solution. The oligonucleotides

thus isolated were labeled with γ-^{32}P-ATP by employing T_4 polynucleotide kinase(New England Biolabs., #201S, USA) and sequenced in accordance with Maxam & Gilbert's method[see: Maxam, A. M. & Gilbert, W., Proc. Natl. Acad. Sci., USA, 74:560-564 (1977)].

Example 4: Ligation of oligonucleotides

100pmole of each oligonucleotide prepared in Example 3, where all the oligonucleotides except for two oligonucleotides(C1 and N1) were phosphorylated at 5' terminal, were placed in Eppendorf tube with the addition of 40μl 0.1M Tris-HCl(pH 7.5). Then, incubation was carried out at 100°C for 3min to denature oligonucleotides, and renaturation was followed by lowering temperature slowly. To the resultant were added 10units of T_4 DNA ligase(New England Biolabs., #202S, USA) and ligation buffer solution, and incubated at 4°C for 12hr and at room temperature for 3hrs, respectively. Then, 7% polyacrylamide gel electrophoresis and autoradiography were followed to determine the ligated hEGF sequence of the invention.

Example 5: Construction of pUE118 and its transformants

pUC18 known in the art(see: Norrander, J. et al, Gene, 26:101(1985)) was digested with SmaI and PstI(New England Biolabs., #141S and #140S, MA, USA; all the restriction enzymes and linkers described hereinafter were purchased from New England Biolabs., MA, USA), and ligated with hEGF gene of the present invention. Ligation was easily performed, based on its nature of blunt 5' terminal and sticky 3' terminal in hEGF gene as designed. Recombinant vector thus obtained was named pUE118[see: Fig. 3].

Then, pUE118 were digested with HpaI, PstI, Bpu1102I, NsiI, MluI, Eco47III and AflII, respectively, and electrophoresis of the gene fragments produced was carried out on 1% agarose gel. As disclosed in Fig. 4, the insertion of

hEGF gene was carried out in an accurate manner and it was confirmed that single restriction site exists for each restriction enzyme. In Fig. 4, Lane 1 is 1kb ladder DNA (BRL, USA); Lanes 2 and 3 are pUE118 digested with HpaI and PstI, respectively; Lane 4 is pUE118 digested doubly with HpaI and PstI; and, Lanes 5-9 are pUE118 digested with Bpu1102I, NsiI, MluI, Eco47III and AflII, respectively.

Competent E. coli JM109 was transformed with the pUE118; and E. coli JM109 thus transformed was deposited with the Korean Culture Center of Microorganisms(KCCM) in the name of Escherichia coli(DW/BT-2040) on April 9, 1993 as deposition No. KCCM 10026.

Example 6: Synthesis of "Omp A leader-universal translation termination-Trp A transcription termination sequence"

Oligonucleotide comprising Omp A leader, universal translation termination and trp A transcription termination sequence designed to have several restriction sites as disclosed in Fig. 5. The oligonucleotide comprises restriction sites of BamHI at 5′ terminal and XbaI at 3′ terminal, and NaeI and PstI between Omp A leader and universal translation termination sequence to assure that N-terminal amino acid sequence is exactly the same as original protein free of additional amino acid sequence.

First of all, Omp A leader-universal translation termination-Trp A transcription termination sequence was synthesized as following 8 oligonucleotides of 31mer or 32mer in a separate manner:

5′ GATCCAAAATTATGAAAAAGACAGCTATCGGC 3′

5′ GATTGCAGTGGCACTGGCTGGTTTCGCTACC 3′

5′ GTAGCGCAGGCCGGCCTGCAGCTTAATTAATT 3′

5′ AAGCAGCCCGCCTAATGAGCGGGCTTTTTTTT 3′

5′ CTAGAAAAAAAAGCCCGCTCATTAGGCGGGCT 3′

5′ GCTTAATTAATTAAGCTGCAGGCCGGCCTGCG 3′
5′ CTACGGTAGCGAAACCAGCCAGTGCCACTGC 3′
5′ AATCGCGATAGCTGTCTTTTTCATAATTTTG 3′

The oligonucleotides were synthesized with automatic Nucleotide Synthesizer(Pharmacia LKB Biotechnology, Uppsala, Sweden) in accordance with solid-phase phosphate triester method[see: Narang, S. A., Synthesis and Applications of DNA and RNA, Academic Press, 1987].

In an analogy to Example 3, the synthesized oligonucleotides were separated from the silica matrix; removal of protecting group and concentration were followed; isolation of oligonucleotides, labeling with γ-^{32}P-ATP, denaturation and renaturation, and ligation of oligonucleotides were performed.

Example 7: Construction of plasmid pDT420

Plasmid pDR540(Pharmacia LKB Biotechnology, #27-4928-01, Upssala, Sweden) was digested with PvuII(#1032), ligated with XbaI linker and digested with XbaI(#145S) and BamHI (#136S) to obtain 2.4kb DNA fragment. Said fragment was isolated from the gel after electrophoresis of digested fragments by employing Geneclean II DNA elution kit(BIO 101 Inc., CA, USA), and ligated with "Omp A leader-universal translation termination-Trp A transcription termination sequence" prepared in Example 6 by T_4 DNA ligase(#202S). E. coli JM101 was transformed with the ligated DNA according to Hanahan's method[see: DNA Cloning Vol.I; A Practical Approach, IRL Press, 109-135(1985)]. From the transformants, recombinant plasmid was isolated and sequenced in accordance with Maxam and Gilbert's method to screen recombinant plasmid containing "OmpA leader-universal translation termination-Trp A transcription termination sequence". The recombinant plasmid thus selected was named pDT420[see: Fig. 6].

Example 8: Construction of expression vector pDE135 and determination of hEGF expression

Double digestion on plasmid pDT420 was made with NaeI (#190S) and PstI(# 140S) to cleave the sequence between Omp A leader and universal translation termination sequence. On the other hand, plasmid pUE118 prepared in Example 5 was digested with HpaI(#105S) and PstI to obtain 0.17kb hEGF gene. hEGF gene thus obtained was ligated with pDT420 cleaved with NaeI and PstI, and the ligated DNA was transformed into E. coli JM101. From the transformants, recombinant plasmid was isolated by alkaline lysis method[Sambrook et al., Molecular Cloning, a laboratory manual, 2nd Ed., Cold Spring Harbor, 1989]. Referring to PstI and HindIII restriction enzyme map, recombinant plasmid containing hEGF gene between Omp A leader and universal translation termination sequence was selected and named pDE135[see: Fig. 7].

E. coli JM101 was transformed with pDE135, and the transformant was cultured in LB media(Luria-Bertani; Molecular Cloning/a laboratory manual, 2nd Ed., CSH, 1989). Then, expression of hEGF was investigated by SDS-PAGE and Western blot analysis. Expression of hEGF was determined by receptor binding assay(see: M. W. Rieman, Peptides, 8:877-885(1987)) employing A431 cell line(ATCC CRL 1555) and commercially available hEGF(Amersham, ARN 5100, UK) as a standard. hEGF was expressed with a yield of 10mg/L after 30hrs cultivation. Under the circumstance, the inventors assumed that the grounds of low efficiency of expression were: the biased transcriptional direction between ampicillin resistant marker and hEGF gene; competition of protein production; and, instability of the expression vector in transformant. Accordingly, the inventors determined to develop an improved hEGF expression cassette to solve the above confronted problems by employing tetracycline resistant marker whose product was not secreted out of cytosol, instead of ampicillin resistant marker.

Example 9: Construction of plasmid pTC108

Plasmid pBR322(Bolivar, F. et al., Gene, 2:95-113 (1977)) was digested with AvaI(#152S), and manipulated with Klenow's fragment(#210S) to have blunt-end. Then, the resultant was digested with EcoRI and followed by electrophoresis on 0.8% agarose gel, and the 1.4kb DNA fragment was obtained from gel by employing Geneclean II DNA elution kit. On the other hand, pUC19(Yanish-Perron, C., et al., Gene, 33:103-119(1985)) was digested with DraI(#129S) and EcoRI to give 1.2kb DNA fragment. The 1.2kb DNA fragment has replication origin of pUC19 to maintain high efficiency of replication and multiple cloning site to ease gene manipulation. 1.4kb and 1.2kb DNA fragments thus obtained were ligated with T_4 DNA ligase, and E. coli JM101 was transformed with the ligated DNA fragment in accordance with Hanahan's method. From the transformants, recombinant plasmid was isolated by alkaline lysis. Referring to EcoRI and AflIII restriction enzyme map, recombinant plasmid containing replication origin of pUC19, multiple cloning site and tetracycline resistant marker was selected and named pTC108[see: Fig. 8].

Example 10: Construction of plasmid pTC226

The inventors introduced par site for the stability of expression vector in transformant and easy separation of plasmids after cell division. Plasmid pSC101(Cohen and Chang, Proc. Natl. Acad. Sci., USA, 70:1293-1297(1973); KCTC 11251) was digested with AvaI(#152S) and electrophoresed on 0.8% agarose gel; and, 3.3kb DNA fragment was obtained therefrom by employing Geneclean II DNA elution kit. DNA fragment thus obtained was manipulated with Klenow's fragment to produce blunt-end, ligated with EcoRI linker (#1020) and digested with EcoRI. The 3.3kb DNA fragment containing sticky end of EcoRI site was digested with HincII(#103S) and electrophoresed on 0.8% agarose gel.

0.37kb DNA fragment containing par site was obtained from electrophoresed gel by employing Geneclean II DNA elution kit. The obtained DNA fragment was ligated to pTC108 digested with EcoRI and SmaI(#141S) by the aid of T_4 DNA ligase, and E. coli JM101 was transformed with the ligated DNA fragment in accordance with Hanahan's method. From the transformants, recombinant plasmid was isolated by alkaline lysis. Referring to EcoRI and PstI restriction enzyme map, recombinant plasmid containing par site was selected and named pTC226[see: Fig 9].

Example 11: Construction of expression vector pTE105

The inventors constructed plasmid vector which is stable in E. coli and is of high efficiency of hEGF expression, by inserting hEGF gene expression cassette of the pDE135 into pTC226 to have same transcriptional direction. Plasmid pTC226 was digested with AflIII, manipulated with Klenow fragment to produce blunt-end, digested with XbaI(#145S) and electrophoresed on 0.8% agarose gel; and, 2.5kb DNA fragment was obtained from electrophoresed gel by employing Geneclean II DNA elution kit. In the same manner, plasmid pDE135 was digested with HindIII(#104S), blunt-ended with Klenow fragment, digested with XbaI and electrophoresed on 1% agarose gel; and, 0.45kb DNA fragment was obtained from electrophoresed gel by employing Geneclean II DNA elution kit. Each isolated DNA fragment, i.e., 2.5kb and 0.45kb DNA fragments, was ligated with T_4 DNA ligase, and E. coli JM101 was transformed with the ligated DNA fragment in accordance with Hanahan's method. From the transformant, recombinant plasmid was isolated by alkaline lysis. Referring to restriction EcoRI and BamHI enzyme map, a recombinant plasmid containing tetracycline resistant marker, par site, and Omp A leader and hEGF gene was selected and named pTE105[see: Fig. 10]. E. coli JM101 transformed with pTE105 was named DW/BT-2042, and deposited with the Korean Culture Collection of Micro-

organisms(KCCM) on April 9, 1993 as deposition No. KCCM 10027.

Example 12: Expression of hEGF in transformants

Transformant E. coli DW/BT-2042(KCCM 10027) was cultured in LB media for 5hrs with the addition of isopropyl β-D-thio-galactoside(IPTG, Sigma I-6758) to the final concentration of 1mM; and, cultures for 19hrs and 25hrs were collected and centrifuged respectively. The supernatants were electrophoresed on 15% polyacrylamide gel(see; H. Schagger et al., Anal. Biochem., 166:368-379(1987)) employing commercially available hEGF(Amersham, ARN 5100, UK) as a standard. Fig. 11A showed that molecular size of hEGF expressed is determined as 6,000 dalton. Western blot analysis(W. N. Burnett, Anal. Biochem., 112:195-203(1981)) was also carried out to confirm that the produced was hEGF[see: Fig. 11B]. In Figs. 11A and 11B, Lane 1 is molecular weight marker(Sigma, #MW-SDS-17S); Lane 2 is the standard hEGF; Lane 3 is the culture for 24hrs without addition of IPTG; Lane 4 is the culture for 30hrs without addition of IPTG; Lane 5 is the culture for 24hrs with addition of IPTG ; and, Lane 6 is the culture for 30hrs with addition of IPTG. The amount of the hEGF in cultures for 24hrs and 30hrs were analyzed quantitatively by hEGF receptor binding assay; and disclosed in Table 1.

As clearly illustrated in Table 1, it was determined that: total hEGF amount of cultivation for 30hrs was 343.5mg/L and most of expressed hEGF was secreted out of cytosol.

Table 1:

Incubation time / sample	24 hours	30 hours
Periplasm	10.5mg/L	9.4mg/L
Culture	213.8mg/L	334.1mg/L
Total	224.3mg/L	343.5mg/L

Example 13: Mass production of hEGF

Transformant E. coli DW/BT-2042(KCCM 10027) was cultured in 4ml of LB media containing 0.5% glucose and 12.5μg/ml of tetracycline at 37°C for 11hrs; and, 400μl of the culture was inoculated on 100ml of the same media and incubated for 11hrs to obtain seed culture. 80ml of seed culture was inoculated to 2L of media containing bacto-trypton 10g, yeast extract 20g, KH_2PO_4 3g, $Na_2HPO_4 \cdot 8H_2O$ 4g, $(NH_4)_2HPO_4$ 2.5g, $CaCl_2 \cdot 2H_2O$ 0.01g, Sigma antifoam A 1ml, glucose 5g and tetracycline 5mg per 1L, and incubated at 30°C. After 4hrs incubation, IPTG was added to the final concentration of 1mM for induction of hEGF and further incubation was carried out for 26hrs.

Example 14: Quantitative assay of produced hEGF

The amount of expressed hEGF was determined by receptor binding assay employing A431 cell line(ATCC CRL 1555), which is modified method of Rieman's[see: Rieman, M.W., Peptides, 8:877-885(1987)] and DiAugustine's[see: DiAugustine, R. P., J. Biol. Chem., 260:2807-2811(1985)]. The A431 cells were mixed with DMEM medium containing calf serum, inoculated on Costa 24well cell culture plate to 4 x 10^5 cells/well and incubated under 5% CO_2-atmosphere at 37°C for 6 or 7 days with the change of new medium every other day. Then, removal of medium and washing with saline phosphate buffer were followed, and cells were immobilized by treatment of 10% formaldehyde for 10min. After the removal of formaldehyde was carried out by washing with saline phosphate buffer, 250μl of receptor binding buffer consisting of 1% BSA, 0.2% sodium azide and saline phosphate buffer was added to every well. To the above, were added standard hEGF solution diluted to 0.01-20ng/20μl or hEGF solution to be determined and ^{125}I-EGF(Amersham, IM196, UK) diluted to 30,000cpm/100μl in a serial manner, and incubation with shaking at 100rpm was followed for 2hrs.

Then, each well was rinsed with receptor binding buffer and bound cells thereon were separated from the well by the incubation with 250μl of cell lysis solution consisting of 0.1N NaOH and 1% SDS. Radioactivity of ^{125}I-EGF bound cells were determined with the aid of γ-scintillation counter (Packard Cobra II, USA).

Example 15: Purification of hEGF

2L of culture prepared in Example 13 was centrifuged (Sorvall™ RC 28S, USA) at 8,000rpm for 30min. The supernatant was loaded on Amberchrom CG71(Tosohass Corp., USA) column(2.5 x 40cm) preequilibrated with 20mM Tris buffer(pH 8.0). Washing was made with 1L of the same buffer; and step elution was carried out with 500ml of 20mM Tris buffer(pH 8.0) containing 40% acetonitrile. Flow rate was controlled at 60cm/hr in the course of loading, washing and elution. The eluted hEGF fraction was stored at 4°C.

350ml of hEGF fraction obtained as above was loaded on Q-Sepharose FF(Pharmacia, USA) column(2.5 x 40cm) preequilibrated with 20mM Tris buffer(pH 8.0) and washed with 500ml of the same buffer. Elution was made with linear gradient of 0M to 0.5M NaCl in 20mM Tris buffer(pH 8.0). In this connection, flow rate was controlled at 55cm/hr during loading, washing and elution. 316.4mg of hEGF was obtained with 80 to 85% of purity.

The hEGF fraction obtained as above was adjusted to pH 6.5 with 20% phosphate solution, and loaded on C_{18} reverse phase column(Waters Delta Prep 4000, USA, 8 x 100mm) and followed by second C_{18} reverse phase column(8 x 100mm). The C_{18} reverse phase columns employed were preequilibrated with 10mM phosphate buffer(pH 6.5); and, flow rate was controlled at 4ml/min. The elution of hEGF was carried out with 10mM phosphate buffer(pH 6.5, buffer A) and 10mM phosphate buffer containing 70% acetonitrile(buffer B). 98% of purity was obtained at the retention time of 29min. Table 2 illustrates HPLC chromatographic conditions for hEGF purification.

Table 2:

Retention time(min)	Flow rate (ml/min)	Buffer ratio (A/B, %)	Gradient type
0	4	0 / 100	-
2	4	74 / 26	linear
20	4	72 / 28	linear
45	4	70 / 30	linear
70	4	66 / 44	linear
75	4	0 / 100	linear
85	4	0 / 100	linear

The homogeneity of purified hEGF in process of purification was determined by SDS-PAGE(Schagger. H. et al., Anal. Biochem., 166:368-379(1987))[see: Fig. 12]. In Fig. 12, Lane A is supernatant obtained by centrifugation of culture; Lane B is proteins isolated by Amberchrom CG71 chromatography; Lane C is proteins isolated by Q-Sepharose FF anion exchange chromatography; and, Lane D is purified hEGF by C_{18} reverse phase HPLC.

The purification step of hEGF is summarized in Table 3.

Table 3:

Purification step	Total volume (ml)	Total hEGF (mg)	Total protein (mg)	Yield (%)	Specific activity (mg hEGF/ mg protein)
Culture	2,000	582.1	5,490	100	0.106
Amberchrom CG 71	350	436.5	997	74.9	0.438
Q-Sepharose FF	300	316.4	414	54.3	0.764
C_{18} HPLC	240	282.3	285	48.4	0.990

282.3mg of purified hEGF was obtained with the yield of 48.4%. Protein concentration was determined by Bradford method(Bradford, M., Anal. Biochem., 72:248(1976)) employing protein quantitative kit(Biorad, #500-0006, USA). The molecular weight of purified hEGF was determined as 6,000

dalton by 15% SDS-PAGE analysis[see: Fig. 13]. In Fig. 13, Lane A is low molecular size weight marker(Biorad, #161-0304); Lane B is purified hEGF; and, Lane C is peptide marker(Sigma, #MW-SDS-17S).

The purity of purified hEGF was analysed in HPLC and represented in Fig. 14. As clearly disclosed in Fig. 14, it was determined that: the purity of hEGF was over 98% and degradation of C-terminal and oxidation of methionine were not occured.

Finally, isoelectric focusing analysis was carried out for pI value determination in the pH range of 4 to 6 ampholite(see: Carfin, D.E., Methods Enzymol., 183:459-475 (1990)) and its result was shown in Fig. 15. In Fig. 15, Lanes A and D are low calibration isoelectric focusing analysis marker(Pharmacia, #17-0472-01, USA); Lanes B and C are 2μg of purified hEGF; and, Lane E is 4μg of purified hEGF. As well be seen in Fig. 15, it was determined that pI of the purified hEGF is 4.55, which is the same pI value of prior art hEGF.

Example 16: Limulus Amebocyte Lysate test

The purified hEGF was lyophilized and followed by Limulus Amebocyte Lysate test(Associates of Cape Code, USA). The result showed that the amount of endotoxin in hEGF was below 0.36EU per 1mg, which was extremely low. Accordingly, hEGF purification process of the invention was determined to be very efficient, while reducing contamination of other celluar protein and endotoxin.

WHAT IS CLAIMED IS:

1. A human epidermal growth factor gene comprising restriction sites of HpaI at 5′ terminal and PstI at 3′ terminal, and Bpu1102I, NsiI, MluI, Eco47III and AflII, whose nucleotide sequence is represented as:

```
5′ GTT AAC AGC GAC TCC GAA TGC CCG CTG AGC CAT GAC GGC
3′ CAA TTG TCG CTG AGG CTT ACG GGC GAC TCG GTA CTG CCG

   TAC TGC CTG CAC GAC GGC GTA TGC ATG TAG ATC GAA GCA
   ATG ACG GAC GTG CTG CCG CAT ACG TAC ATC TAG CTT CGT

   CTG GAC AAA TAC GCG TGC AAC TGT GTT GTT GGC TAC ATC
   GAC CTG TTT ATG CGC ACG TTG ACA CAA CAA CCG ATG TAG

   GGC GAG CGC TGT CAG TAC CGT GAC CTT AAG TGG TGG GAA
   CCG CTC GCG ACA GTC ATG GCA CTG GAA TTC ACC ACC CTT

   CTG CGC TGATAACCTGCA 3′
   GAC GCG ACTATTGG     5′
```

2. A recombinant vector pUE118, which comprises the nucleotide sequence of claim 1.

3. E. coli JM109(KCCM 10026) transformed with the recombinant vector of claim 2.

4. An expression vector pTE105 for human epidermal growth factor comprising an expression cassette consisting of Omp A leader sequence, translation termination sequence and transcription termination sequence and hEGF gene, wherein the expression cassette is under transcriptional regulation by tac promoter.

5. The expression vector pTE105 of claim 4, which comprises replication origin and regulation site originated

from pUC19.

6. The expression vector pTE105 of claim 4, which comprises par site for its stabilization in E. coli.

7. The expression vector pTE105 of claim 4, which comprises restriction sites of NaeI and PstI between Omp A leader sequence and universal translation termination sequence.

8. E. coli JM101(KCCM 10027) transformed with the expression vector of claim 4.

9. A process for preparing human epidermal growth factor from the culture of E. coli JM101(KCCM 10027) of claim 8.

10. A process for preparing human epidermal growth factor, which comprises culturing E. coli JM101(KCCM 10027) of claim 8.

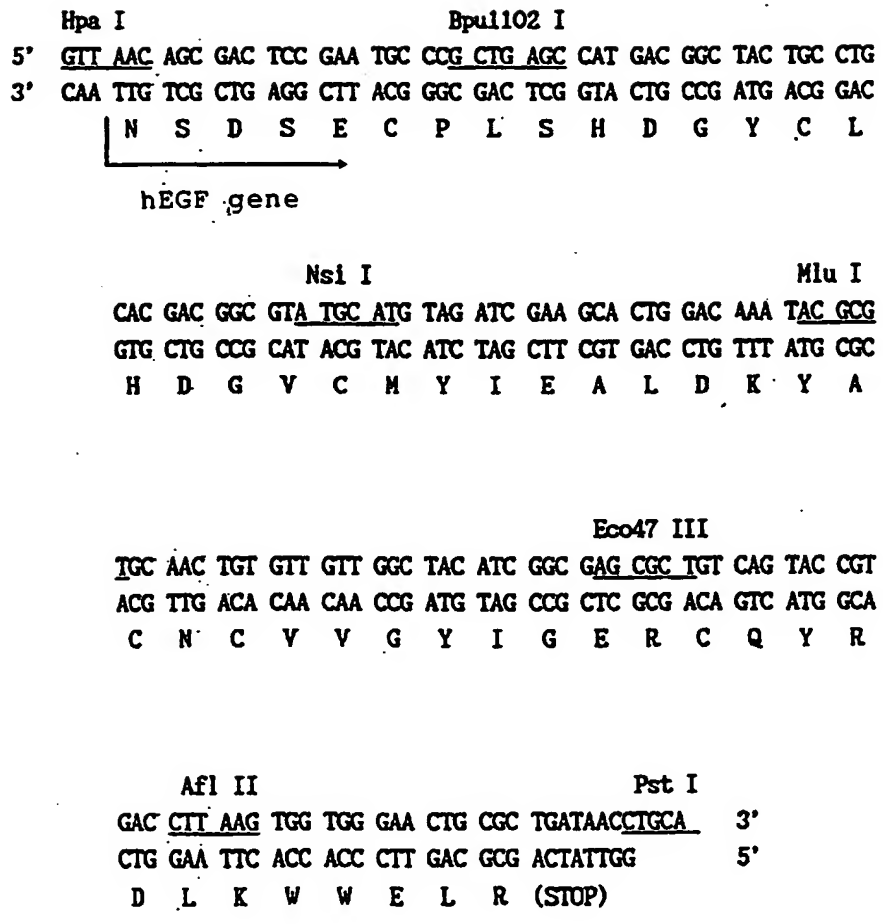

Hpa I
Bpu1102 I
5' GTT AAC AGC GAC TCC GAA TGC CCG CTG AGC CAT GAC GGC TAC TGC CTG
3' CAA TTG TCG CTG AGG CTT ACG GGC GAC TCG GTA CTG CCG ATG ACG GAC
N S D S E C P L S H D G Y C L
hEGF gene
Nsi I
Mlu I
CAC GAC GGC GTA TGC ATG TAG ATC GAA GCA CTG GAC AAA TAC GCG
GTG CTG CCG CAT ACG TAC ATC TAG CTT CGT GAC CTG TTT ATG CGC
H D G V C M Y I E A L D K Y A
Eco47 III
TGC AAC TGT GTT GTT GGC TAC ATC GGC GAG CGC TGT CAG TAC CGT
ACG TTG ACA CAA CAA CCG ATG TAG CCG CTC GCG ACA GTC ATG GCA
C N C V V G Y I G E R C Q Y R
Afl II
Pst I
GAC CTT AAG TGG TGG GAA CTG CGC TGATAACCTGCA 3'
CTG GAA TTC ACC ACC CTT GAC GCG ACTATTGG 5'
D L K W W E L R (STOP)

FIG. 1

C1 , 30 mer : 5' GTTAACAGCGACTCCGAATGCCCGCTAGC 3'

C2 , 35 mer : 5' CATGACGGCTACTGCCTGCACGACGGCGTATGCAT 3'

C3 , 29 mer : 5' GTACATCGAAGCACTGGACAAATACGCGT 3'

C4 , 39 mer : 5' GCAACTGTGTTGTTGGCTACATCGGCGAGCGCTGTCAGT 3'

C5 , 41 mer : 5' ACCGTGACCTTAAGTGGTGGGAACTGCGCTGATAACCTGCA 3'

N1 , 29 mer : 5' GGTTATCAGCGCAGTTCCCACCACTTAAG 3'

N2 , 38 mer : 5' GTCACGGTACTGACAGCGCTCGCCGATGTAGCCAACAA 3'

N3 , 29 mer : 5' CACAGTTGCACGCGTATTTGTCCAGTGCT 3'

N4 , 36 mer : 5' TCGATGTACATGCATACGCCGTCGTGCAGGCAGTAG 3'

N5 , 38 mer : 5' CCGTCATGGCTCAGCGGGCATTCGGAGTCGCTGTTAAC 3'

FIG. 2A

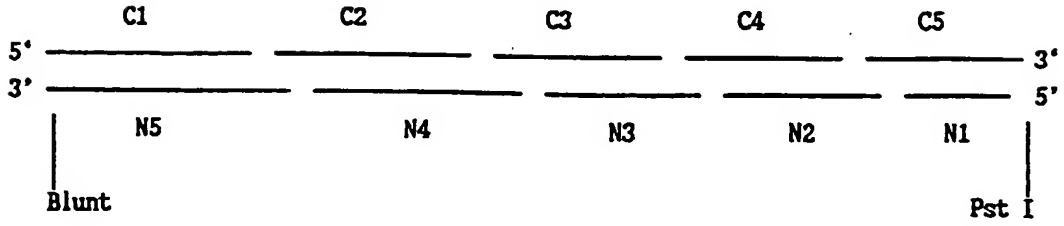


FIG. 2B

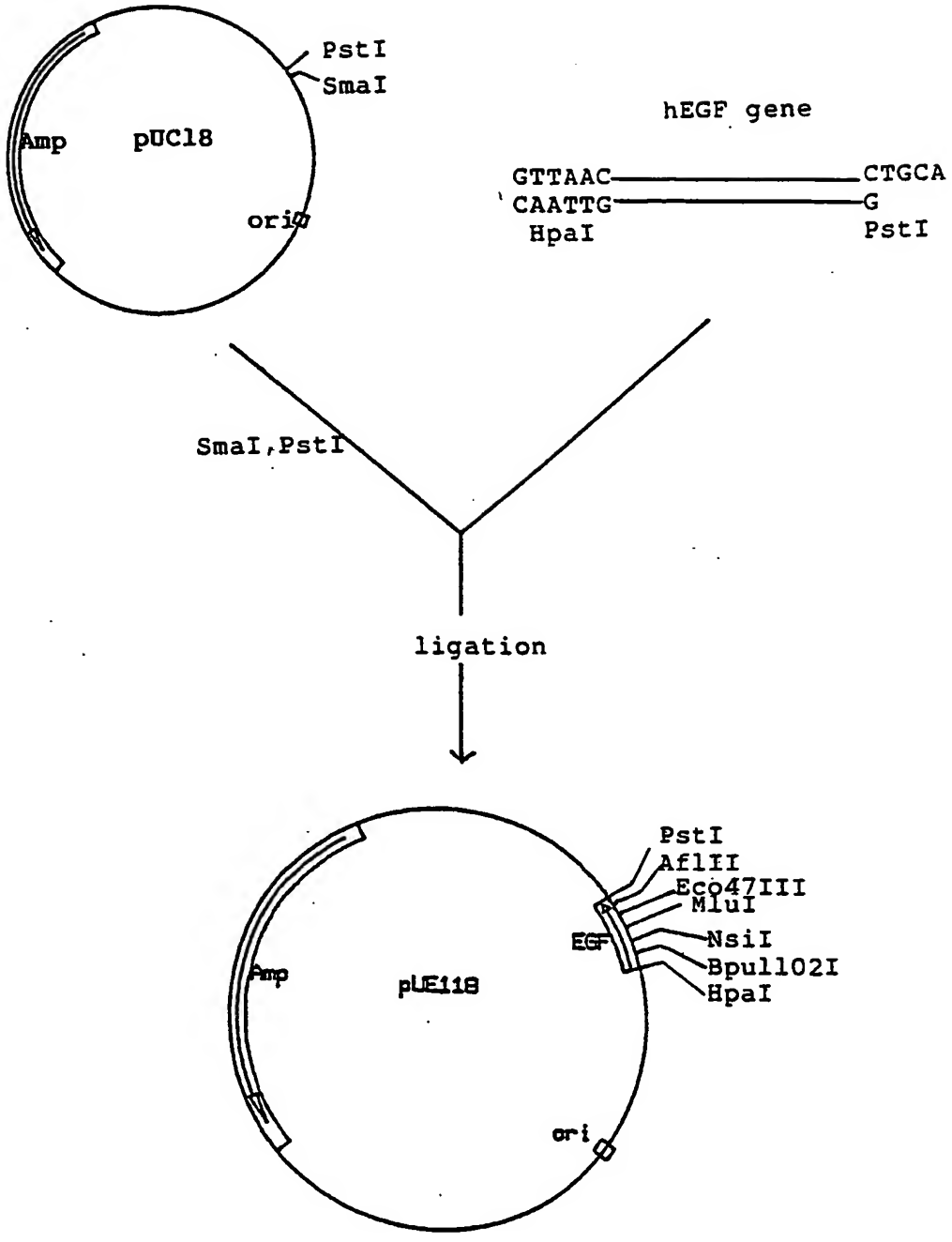

PstI
SmaI
Amp
pUC18
ori
hEGF gene
GTTAAC
CTGCA
CAATTG
G
HpaI
PstI
SmaI,PstI
ligation
PstI
AflII
Eco47III
MluI
EGF
NsiI
Bpu1102I
HpaI
Amp
pUE118
ori

FIG. 3

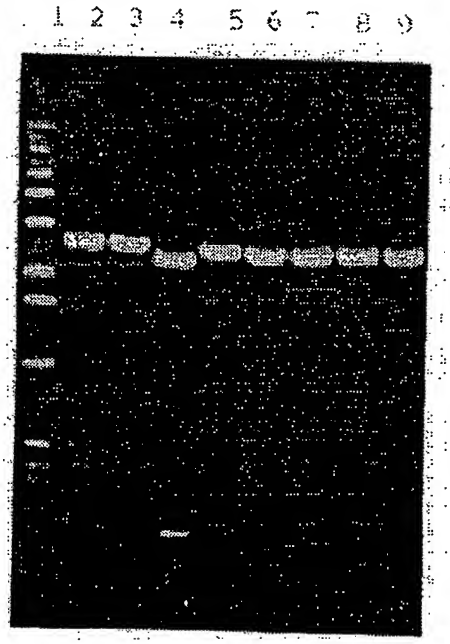


FIG. 4

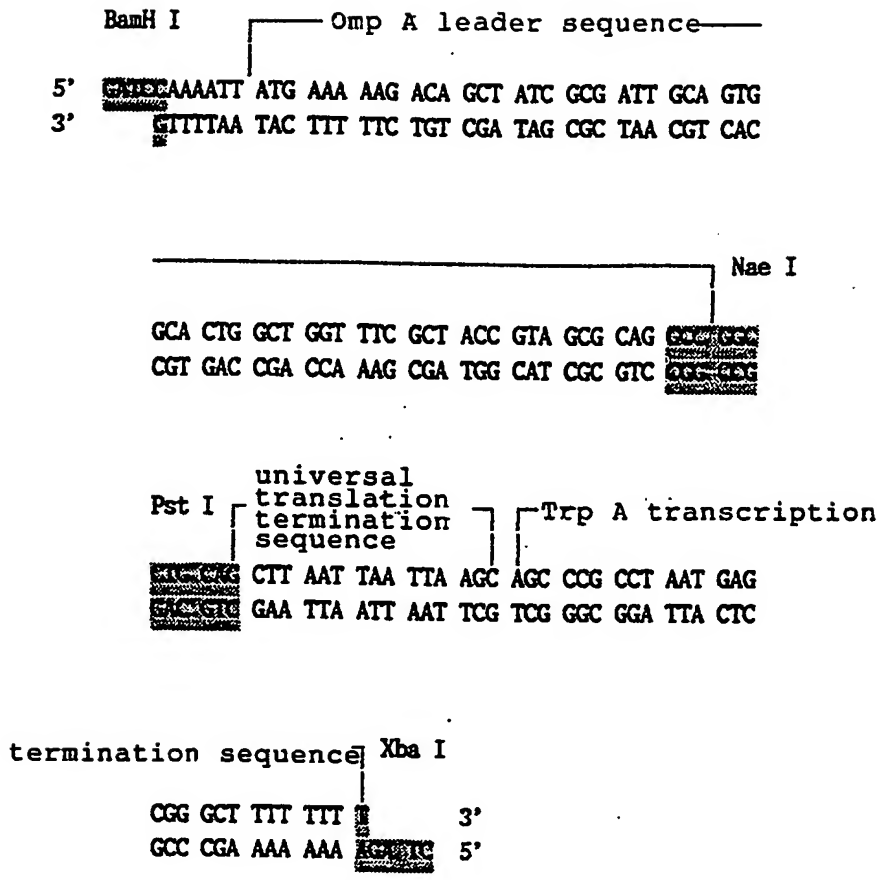

BamH I
Omp A leader sequence
5' GATCCAAAATT ATG AAA AAG ACA GCT ATC GCG ATT GCA GTG
3' GTTTTAA TAC TTT TTC TGT CGA TAG CGC TAA CGT CAC
Nae I
GCA CTG GCT GGT TTC GCT ACC GTA GCG CAG GCC GGC
CGT GAC CGA CCA AAG CGA TGG CAT CGC GTC CGG CCG
Pst I
universal translation termination sequence
Trp A transcription
CTG CAG CTT AAT TAA TTA AGC AGC CCG CCT AAT GAG
GAC GTC GAA TTA ATT AAT TCG TCG GGC GGA TTA CTC
termination sequence
Xba I
CGG GCT TTT TTT T 3'
GCC CGA AAA AAA AGA TC 5'

FIG. 5

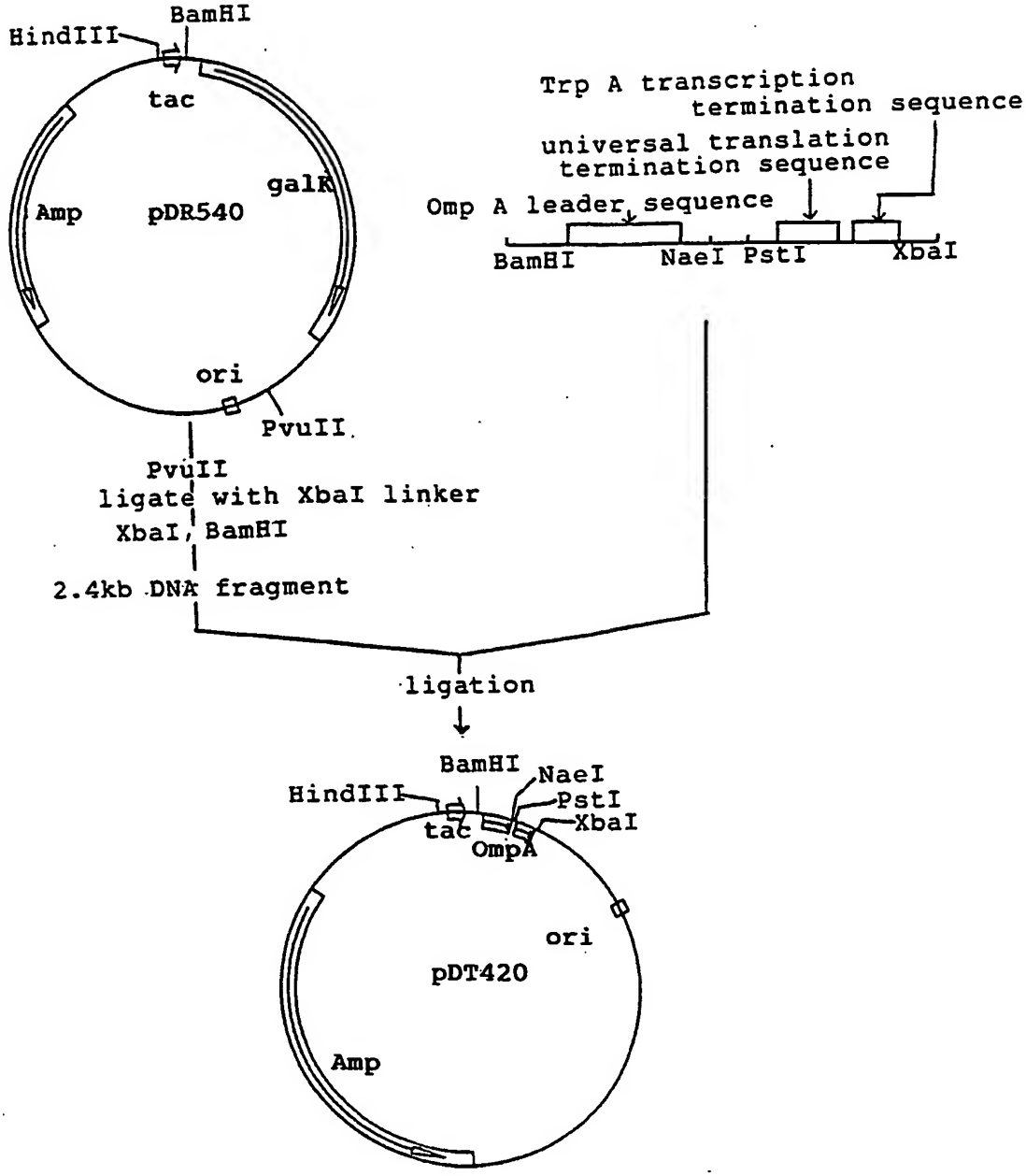
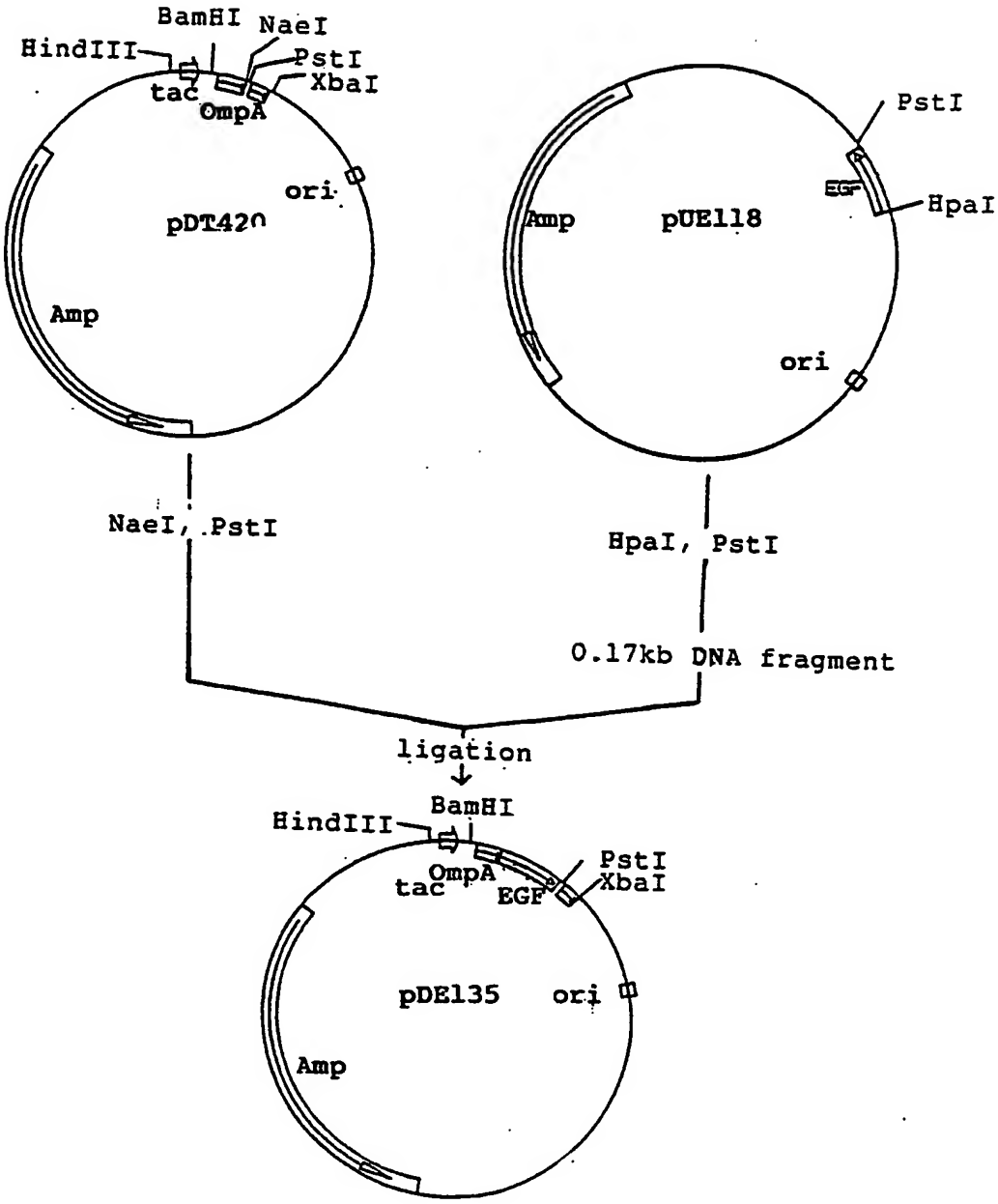

HindIII
BamHI
tac
galK
Amp
pDR540
ori
PvuII
PvuII
ligate with XbaI linker
XbaI, BamHI
2.4kb DNA fragment
Trp A transcription
termination sequence
universal translation
termination sequence
Omp A leader sequence
BamHI
NaeI
PstI
XbaI
ligation
BamHI
NaeI
HindIII
PstI
XbaI
tac
OmpA
ori
pDT420
Amp

FIG. 6


BamHI
NaeI
HindIII
PstI
XbaI
tac
OmpA
ori
pDT420
Amp
PstI
EGF
HpaI
Amp
pUE118
ori
NaeI, PstI
HpaI, PstI
0.17kb DNA fragment
ligation
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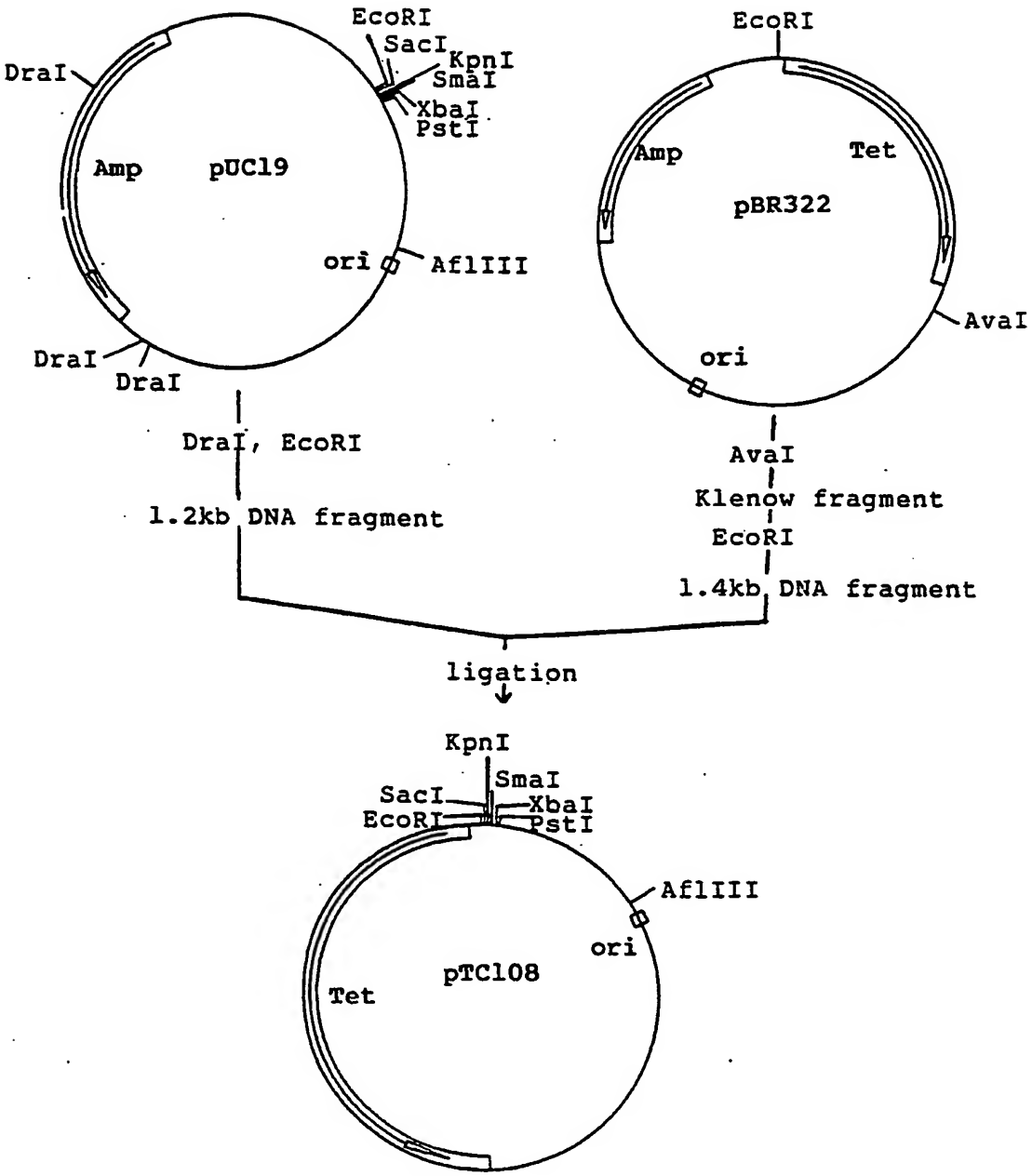
HindIII
PstI
XbaI
OmpA
tac
EGF
pDE135
ori
Amp

FIG. 7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SacI
KpnI
SmaI
XbaI
PstI
DraI
Amp
pUC19
ori
AflIII
DraI
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DraI, EcoRI
1.2kb DNA fragment
EcoRI
Amp
Tet
pBR322
AvaI
ori
AvaI
Klenow fragment
EcoRI
1.4kb DNA fragment
ligation
KpnI
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XbaI
EcoRI
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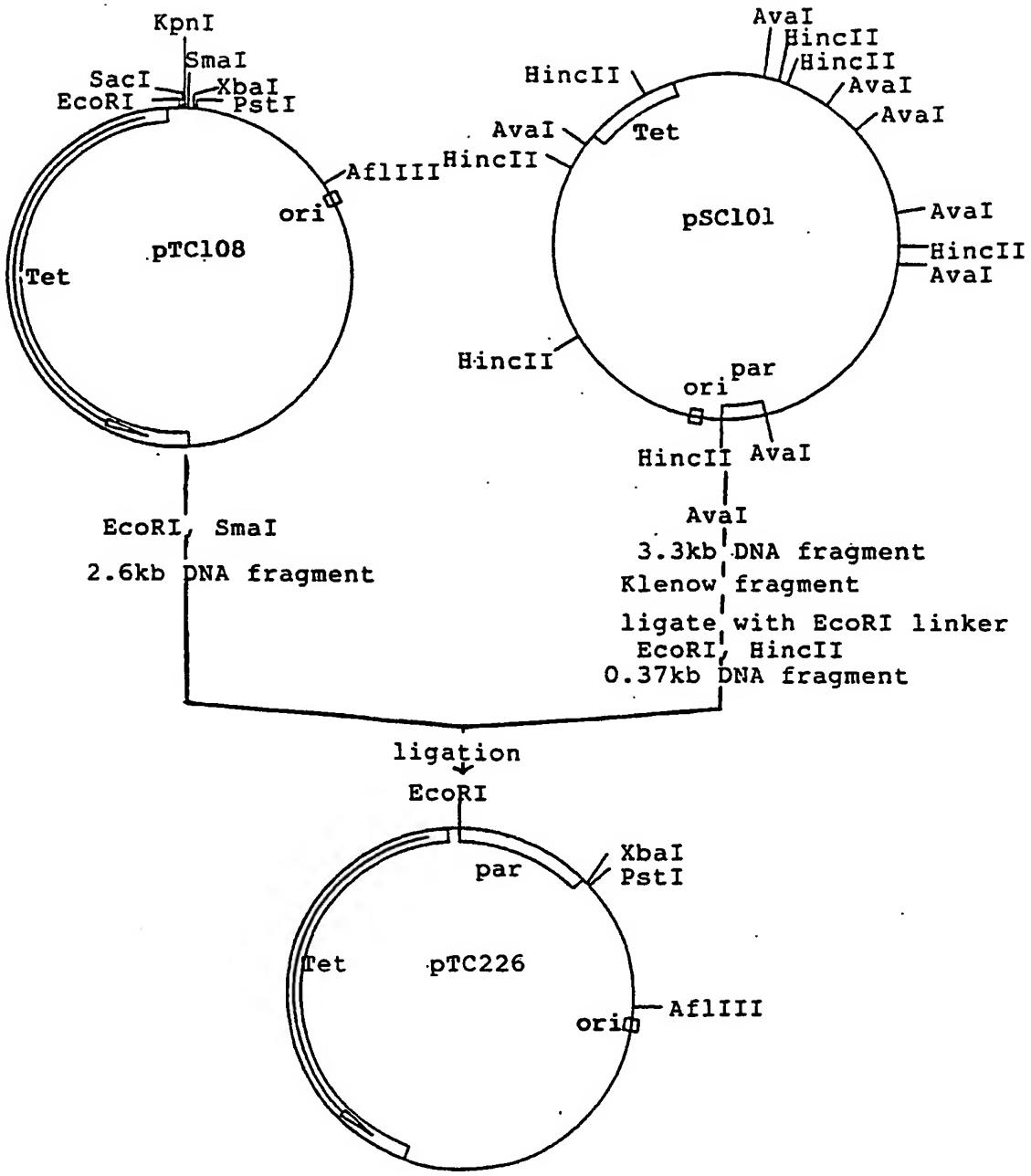
ori
pTC108
Tet

FIG. 8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SmaI
SacI
XbaI
EcoRI
PstI
AflIII
ori
pTC108
Tet
EcoRI, SmaI
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AvaI
HincII
HincII
AvaI
AvaI
HincII
Tet
AvaI
HincII
pSC101
AvaI
HincII
AvaI
HincII
ori
par
HincII AvaI
AvaI
3.3kb DNA fragment
Klenow fragment
ligate with EcoRI linker
EcoRI, HincII
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ligation
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par
XbaI
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Tet
pTC226
ori
AflIII

FIG. 9

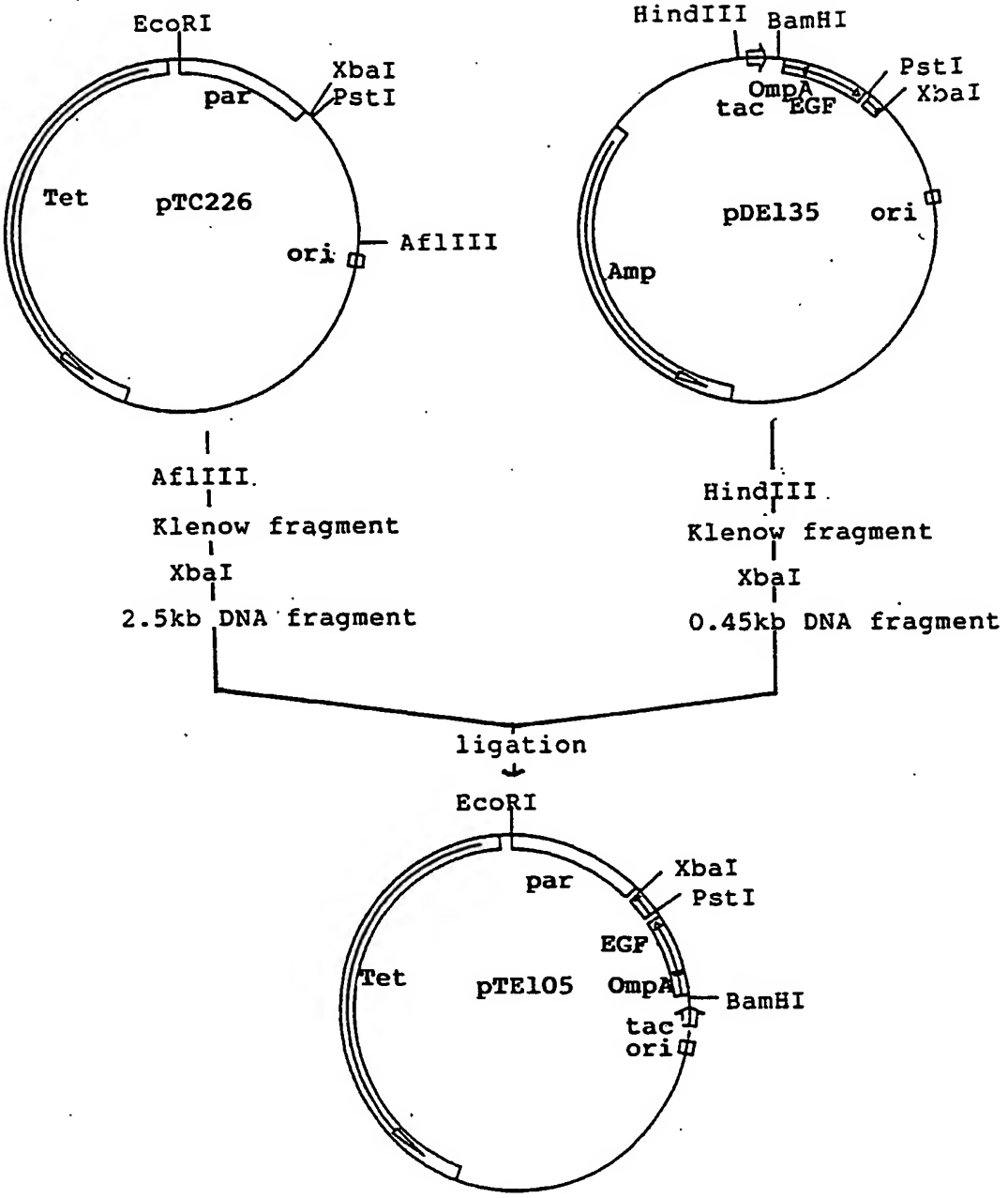
EcoRI
XbaI
PstI
par
Tet
pTC226
ori
AflIII
HindIII
BamHI
PstI
XbaI
OmpA
tac
EGF
pDE135
ori
Amp
AflIII
Klenow fragment
XbaI
2.5kb DNA fragment
HindIII
Klenow fragment
XbaI
0.45kb DNA fragment
ligation
EcoRI
par
XbaI
PstI
EGF
Tet
pTE105
OmpA
BamHI
tac
ori

FIG. 10

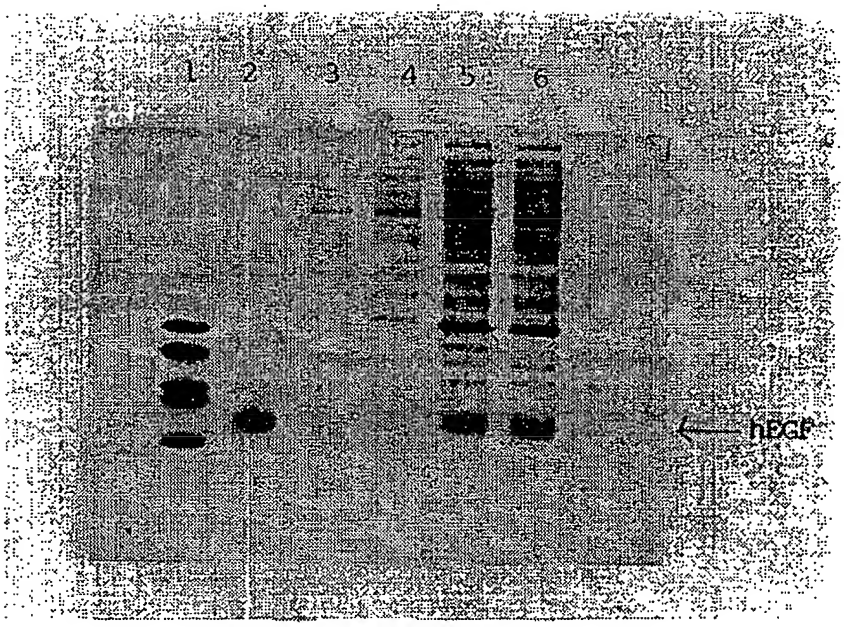

FIG. 11A

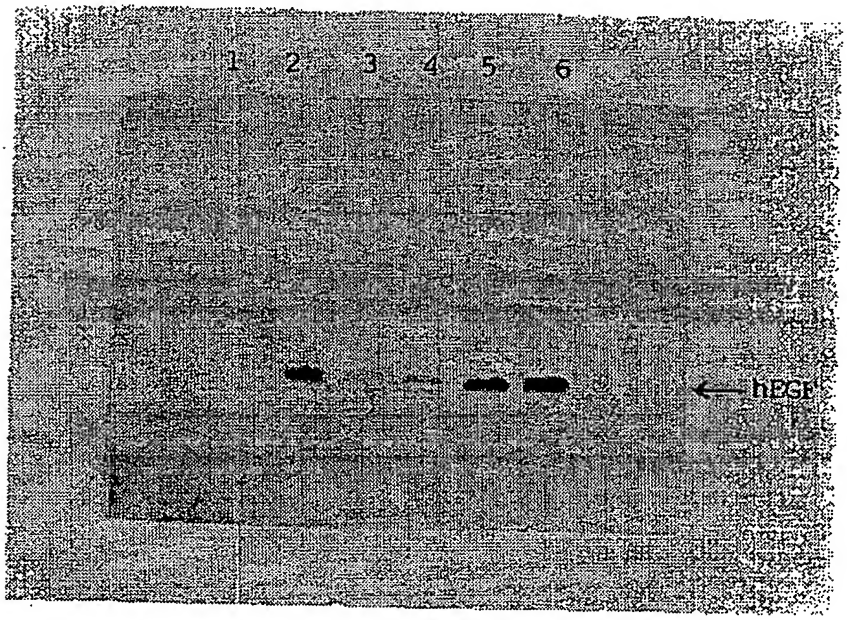

FIG. 11B

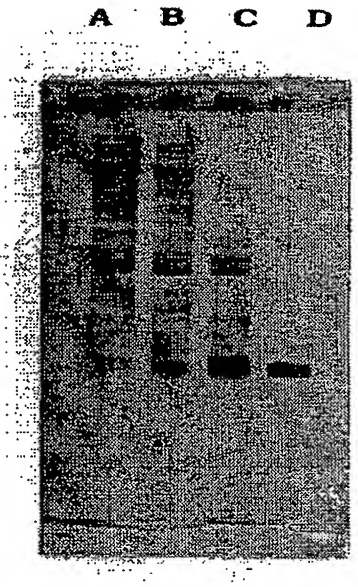

FIG. 12

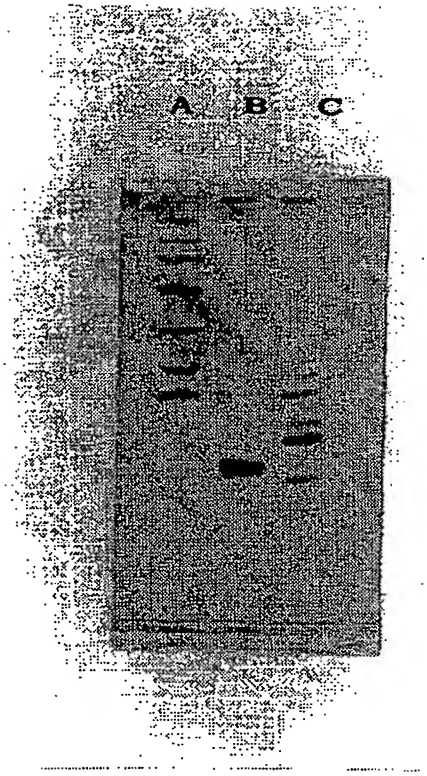

FIG. 13

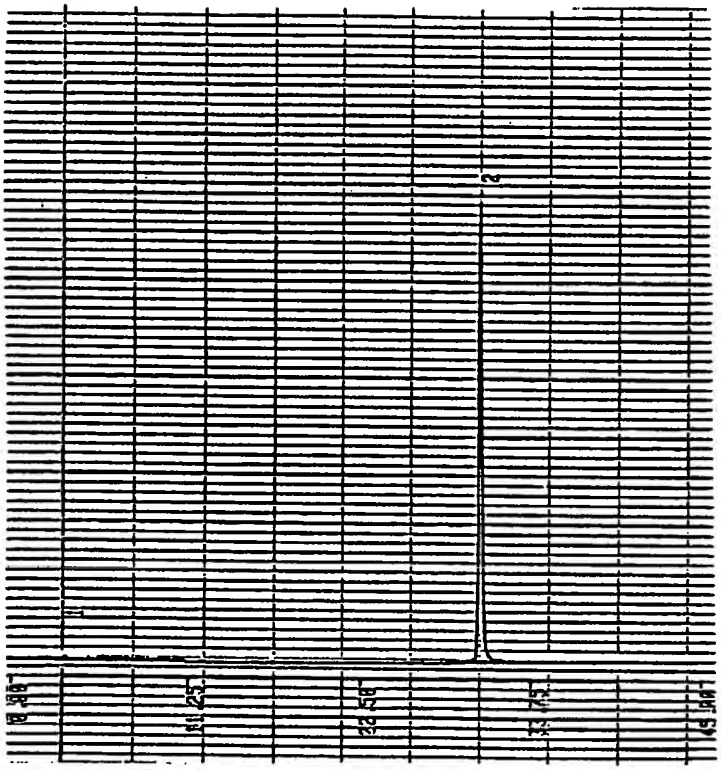

FIG. 14

A B C D E

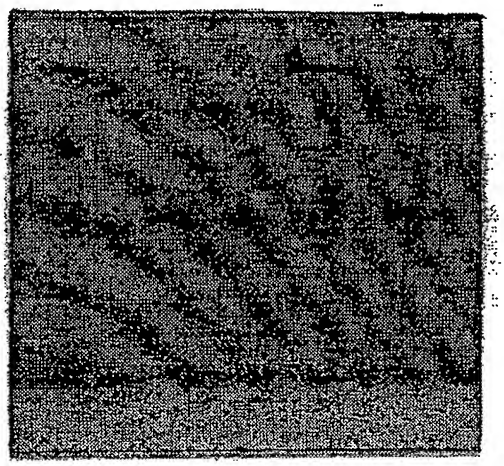

FIG. 15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 94/00036

A. CLASSIFICATION OF SUBJECT MATTER

IPC[5]: C 12 N 15/18, 1/21//(C 12 N 1/21; C 12 R 1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[5]: C 12 N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP, A2, 0 335 400 (HITACHI) 4 October 1989 (04.10.89), claims.	1,3,4,8-10
A	EP, A2, 0 177 915 (TAKEDA CHEMICAL INDUSTRIES) 16 April 1986 (16.04.86), claims.	1,3,4,8-10

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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Date of the actual completion of the international search	Date of mailing of the international search report
22 July 1994 (22.07.94)	11 August 1994 (11.08.94)

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INTERNATIONAL SEARCH REPORT
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International application No.
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